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J Vet Res 68, 395-400, 2024
DOI:10.2478/jvetres-2024-0040

How does adulteration of wax foundation affect phenoloxidase and lysozyme activities as selected parameters of immunity in *Apis mellifera*?

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Received: December 18, 2023 *Accepted: July 24, 2024*

Abstract

Introduction: The adulteration of wax foundation is, for many reasons, a growing problem of modern beekeeping not only in Europe but also around the world. Wax foundation contaminated with stearin addition leads to a brood die-off, while paraffin addition negatively affects the strength of combs. It is tenable that such adulterated wax foundation reduces bees' immunity. The aim of the study was to determine the activities of two bee immune enzymes, lysozyme and phenoloxidase, in the haemolymph of worker bees which had emerged from combs with wax foundations contaminated with stearin or paraffin. **Material and Methods:** Combs built with stearin- or paraffin-adulterated wax (both adulterants at concentrations of 10%, 30% or 50%) or pure wax (0% adulterated) foundations were placed in the colonies, one for each adulterant and percentage. The workers were marked upon emergence from these combs and those bees were introduced into one strong colony per adulterant and percentage. Phenoloxidase and lysozyme activities were determined in the haemolymph of 1-, 7- and 14-day-old workers. **Results:** The higher the concentrations of stearin and paraffin in the wax foundation, the lower the phenoloxidase activities were. These activities increased with the bee age. In contrast, the trends in lysozymes were opposite. Paraffin seems to be less toxic than stearin. **Conclusion:** Adulteration of wax foundation with even a small amount of stearin or paraffin has negative effects on the functioning of the bee.

Keywords: wax adulteration, stearin, paraffin, humoral immunity, phenoloxidase, lysozyme.

Introduction

The worldwide phenomenon of colony collapse disorder (or honey bee colony depopulation syndrome) is a constant cause for concern and its occurrence is escalating in many countries (31). The reasons for this phenomenon may be the stronger negative impact of bee diseases caused by various pathogens (22, 32), plant protection products (18, 32), and inappropriate apiary management methods (25). Chęć *et al.* (5) showed that adulteration of wax substrates may be one of its main causes. The worldwide scarcity and high price of natural honey-bee wax and the lack of procedures for routine quality control of manufactured wax substrates lead to the downgrading of the quality of this raw material through the addition of cheaper substances to it. Stearin and paraffin are among the most commonly used wax additives in the beekeeping industry (4). As early as in 1959, Taranov (30) observed that reared broods are smaller and colonies thus weaker if the quality of wax used for construction of wax foundations is poor. In 2018, the Directorate-General for Health and Food Safety of the European Commission reported that adulteration of wax with paraffin, stearin or palm oil has a negative impact on bee colonies. A content level of 15–35% stearic acid in wax foundation causes the deaths of up to 71% of larvae reared in combs built on such a wax foundation (6). Chęć *et al.* (5) showed that wax substrate with stearin addition leads to a brood die-off and as a result, the beekeeper sees the brood scattered. Moreover, the number of capped brood cells decreases with increasing stearin content, jeopardising the survival of the pupae in such cells. For example, 3.9% stearin addition to the wax foundation cut brood survival by 67%, compared to

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brood survival from larvae in pure wax. Larger 30% and 50% stearin additions to the wax foundation diminished brood survival by 87% and 92%, respectively, compared in the same way. The addition of paraffin to wax foundation does not affect the brood but significantly reduces the mechanical strength of the combs; therefore, the purchase and installation of paraffin-adulterated wax incurs significant losses in honey production (5).

Many publications (2, 28, 29) focused on understanding the physicochemical and organoleptic properties of wax and on developing methods for detection of beeswax adulteration, which is also important, but neglected to consider the impact of adulteration on the physiology and immunity of bees. A complex array of apian defences include reactions in the cellular and humoral immune systems. Two of the most important enzymes that determine these processes are phenoloxidase (PO) and lysozyme (LZ). Phenoloxidase belongs to the group of tyrosinases whose main function is the oxidation of phenols. The enzyme occurs as an inactive zymogen (prophenoloxidase – proPO) and is converted into active PO when necessary. Prophenoloxidase is activated by amphiphilic lipids (such as lysolecithin) and damaged cells in the bee, and also by β-1,3-glucans, lipopolysaccharides and peptidoglycans from pathogens. In invertebrates, active PO catalyses reactions in the melanisation and sclerotisation pathways that seal and protect the organisms against the ingress of pathogens and other harmful factors. Activation of these processes in bees is essential for their health and homeostasis (1, 8, 12, 24, 27). The level of the second of the main compounds, LZ (N-acetylmuramylhydrolase; N-acetylmuramide glycanhydrolase; muramidase) is a criterion for determining the degree of resistance of the bees (13). Lysozyme hydrolyses the β-1,4-glycosidic linkage of murein-peptidoglycan, which is a cell wall component in bacteria (34). The PO and LZ concentrations depend on a number of factors: the species, breed, sex and age of the bee, the functions it performs, the protein content in its food, stimulators, environmental status, exposure to pathogenic microorganisms and parasites, *etc*. (16).

It can be supposed that bees which emerge from cells from combs formed on a stearin- or paraffin-adulterated wax foundation will have a weakened immune system, and that these bees will not be able to rebuild their immune potential. These suppositions follow from another – that the activities of PO and LZ in bees will be disturbed as a result of the adulterations. Therefore, the aim of the experiment was to determine the effect of stearin and paraffin addition to wax on these enzyme activities in the haemolymph of bees at different ages.

Material and Methods

Design of the experiment. The experiment was carried out at the apiary and laboratory of the University of Life Sciences in Lublin. Three identical sets of wax combs, each set with a comb containing 100% pure beeswax (control, C) and one comb per percentage contamination containing wax with the addition of 10%, 30% or 50% paraffin (PA; groups PA10%, PA30% and PA50%) or stearin (ST; groups ST10%, ST30% and ST50%) were prepared, to make a total of 21 combs. These were made ready according to the method published by Chęć *et al.* (5).

The stearin adulteration was with Radiacid 444 (Standard, Lublin, Poland), a solid mixture of stearic and palmitic acids. Its melting temperature was 67°C. Using gas chromatography–mass spectrometry (GC-MS), it was determined that the stearin consisted of 61% palmitic acid and 39% stearic acid, meaning that 3.8%, 11.4% and 19.0% of the adulteration in the wax to which this was added was stearic acid and 6.2%, 18.6% and 31% of the adulteration was palmitic acid. The paraffin adulteration was with Kunlun 56–58 (Standard), a mixture of solid alkanes containing from 16 to 48 carbon atoms per molecule and separated from heavy fractions of crude oil with boiling points over 350°C. Its melting temperature was 59°C. A GC-MS analysis showed that the paraffin was composed of saturated hydrocarbons (alkanes) with hentriacontane being the largest component and containing 31 carbon atoms in a chain (14%). The composition of paraffin and beeswax in terms of the content of long-chain alkanes is presented by Chęć *et al.* (5). The palmitic acid concentration in the reference beeswax used for the analyses was 4.2%.

The same type of comb was placed in each of three colonies of *Apis mellifera carnica*; colonies with similar strength and structure were selected and they totalled 21 (7 types of comb as experimental groups × 3 colonies). The queen was taken from each of these colonies and held for 24 h in a queen-excluder comb cage containing the empty experimental comb for egg laying. The queens from all groups were sisters. Twenty days after the eggs had been laid in them, the capped brood combs were removed from the colonies and each was placed in a separate isolator and next in an incubator until the worker bees emerged. Haemolymph was collected from ten 1-day-old workers from each comb in the group (10 workers × 1 comb from each of 3 co-grouped colonies × 7 groups). One hundred workers which had emerged from the combs with the studied wax foundations from each colony were marked using POSCA PC-3M pens (Uni Mitsubishi Pencil, Tokyo, Japan), giving a total number of marked workers of 2,100 (100 workers × 1 comb from each of 3 co-grouped colonies × 7 groups). Each group was marked with a different colour. All the workers from a group were introduced into one strong colony. Then from 30 workers aged 7 and 14 days in each group (30 workers × 2 age cohorts × 7 groups), haemolymph was collected according to the method published by Łoś and Strachecka (14). An end-to-end glass capillary of 20 μL capacity without anticoagulant (Medlab Products, Raszyn, Poland) was individually inserted between the third and fourth tergite of living workers. Haemolymph volumes were separately measured in each capillary. Haemolymph from one bee was collected into a sterile Eppendorf tube containing

25 μL of ice-cooled 0.6% NaCl. A total of 630 haemolymph samples (30 workers × 3 age cohorts × 7 groups) were collected including those taken from day-old workers. The haemolymph solution was immediately refrigerated at −40°C for further biochemical analyses.

Phenoloxidase activities were determined in haemolymph samples according to the method of Park *et al.* (19) as modified by Ptaszyńska *et al.* (21). A 2 μL volume of the haemolymph sample was combined with 18 μL of tris-buffered saline containing 5 mM $CaCl_2$ in a well of a 96-well plate to give a final sample volume of 20 μL. After 20 min of incubation at room temperature, 180 μL of 2 mM L-dihydroxyphenylalanine in 50 mM sodium phosphate at pH 6.5 was added. Phenoloxidase activity was determined spectrophotometrically on the basis of the amount of melanin formed over 60 min, with reading of the solution for absorbance at 490 nm at 2-min intervals using a microtitre plate reader (PowerWave XS Microplate Spectrophotometer; BioTek, Winooski, VT, USA). The PO activity was determined for each haemolymph sample in each group.

Lysozyme activities were determined in haemolymph samples according to the method of Ptaszyńska *et al.* (20). Each well in an agar plate was filled with 7 μL of haemolymph sample, and after 24 h incubation at 28°C the *Micrococcus lysodeikticus* ATCC No. 4698 (Cat. no. M3770; Sigma-Aldrich, St. Louis, MO, USA) lytic zones were measured. Each haemolymph sample was tested in three repeats. Sterile saline solution was used as the negative control. The activity of lysozyme was calculated from a standard curve made with egg white lysozyme IUBMB EC 3.2.1.17 (Cat. no. L6876; Sigma-Aldrich).

Statistical analysis. The results were analysed statistically using Statistica software version 13.3 for Windows (TIBCO Software, Palo Alto, CA, USA). The distribution of the data was analysed with the Shapiro–Wilk test.

For each age cohort (1, 7 and 14 days), the influence of the levels of paraffin and stearin content in the wax (0%, 10%, 30% or 50%) on the activities of PO and LZ was assessed separately. The influence of age on the activities of PO and LZ in the control group and for individual levels of PA and ST adulteration was also assessed. The distribution of these data was not normal; therefore, they were analysed with the Kruskal–Wallis test.

Within each age cohort, the activities of PO and LZ in the control group were compared with their activities at particular levels of adulteration of the bonds with paraffin and stearin (10%, 30%, 50%) using the Mann–Whitney U test because the data were not normally distributed. Also the Mann–Whitney U test was used to compare the activities of PO and LZ between age cohorts in the control group and groups with specific levels of wax adulteration with paraffin and stearin.

Results

The level of adulteration with paraffin and also with stearin had a statistically significant effect on PO and LZ activities in all age cohorts of worker bees (Table 1). Also the effect of age was statistically significant in the control group and for all adulteration levels with paraffin and stearin (Table 2). The activities of PO and LZ in the control group increased with the age of workers. Phenoloxidase activities were always the highest and LZ activities the lowest in the control group (Fig. 1).

The PO activities in the ST and PA groups decreased with age, and the greater the wax adulteration, the lower the PO activities were. The LZ activities increased with age of workers in each group, and the greater the wax adulteration, the higher the LZ activities were.

In each age group, the PO activities for individual levels of adulteration were higher in PA groups than in ST groups (Fig. 1A; significant difference was found for 7-day-old workers from adulteration level 10% and 30% broods at P-value ≤ 0.01 and for 14-day-old workers from adulteration level 10% for P-value ≤ 0.01, 30% and 50% for P-value ≤ 0.01). Lysozyme activities, in contrast, were lower (Fig. 1B; significant difference was found for 1-day-old and 7-day-old workers from all adulteration level broods for P-value ≤ 0.01; 14-day-old workers, 10% for P-value ≤ 0.01).

Table 1. Effect of adulteration levels of comb wax with paraffin and stearin (0%, 10%, 30% or 50%) in individual age cohorts of *A. mellifera* worker bees (1, 7 and 14 days) on phenoloxidase and lysozyme activities in haemolymph

Age of workers	Effect of adulteration level			
	Paraffin		Stearin	
	phenoloxidase activities	lysozyme activities	phenoloxidase activities	lysozyme activities
1 day	H_3 = 73.31 P-value = 0.0000	H_3 = 99.86 P-value = 0.000	H_3 = 111.57 P-value = 0.000	H_3 = 111.59 P-value = 0.000
7 days	H_3 = 111.58 P-value = 0.000	H_3 = 110.83 P-value = 0.000	H_3 = 111.59 P-value = 0.000	H_3 = 111.58 P-value = 0.000
14 days	H_3 = 111.58 P-value = 0.000	H_3 = 111.57 P-value = 0.000	H_3 = 111.58 P-value = 0.000	H_3 = 110.58 P-value = 0.000

H – value of statistics for the Kruskal–Wallis test; n = 630 (7 groups × 30 workers × 3 age cohorts); the effect of the adulteration level on individual age cohorts of workers was significant at P-value ≤ 0.01

Table 2. Effect of age of *A. mellifera* worker bees (1, 7 and 14 days) from broods from combs with one of four adulteration levels of wax with paraffin and stearin (0%, 10%, 30% or 50%) on phenoloxidase and lysozyme activities in haemolymph

Activities	Effect of age						
	Control	Paraffin			Stearin		
	0%	10%	30%	50%	10%	30%	50%
phenoloxidase	H_2 = 79.13 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.12 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.12 P-value = 0.0000
lysozyme	H_2 = 79.14 P-value = 0.0000	H_2 = 79.13 P-value = 0.0000	H_2 = 79.13 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.13 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000	H_2 = 79.14 P-value = 0.0000

H – value of statistics for the Kruskal–Wallis test; n = 90 (3 age cohorts × 30 worker bees); the effect of age on individual adulteration level groups was significant at P-value ≤ 0.01

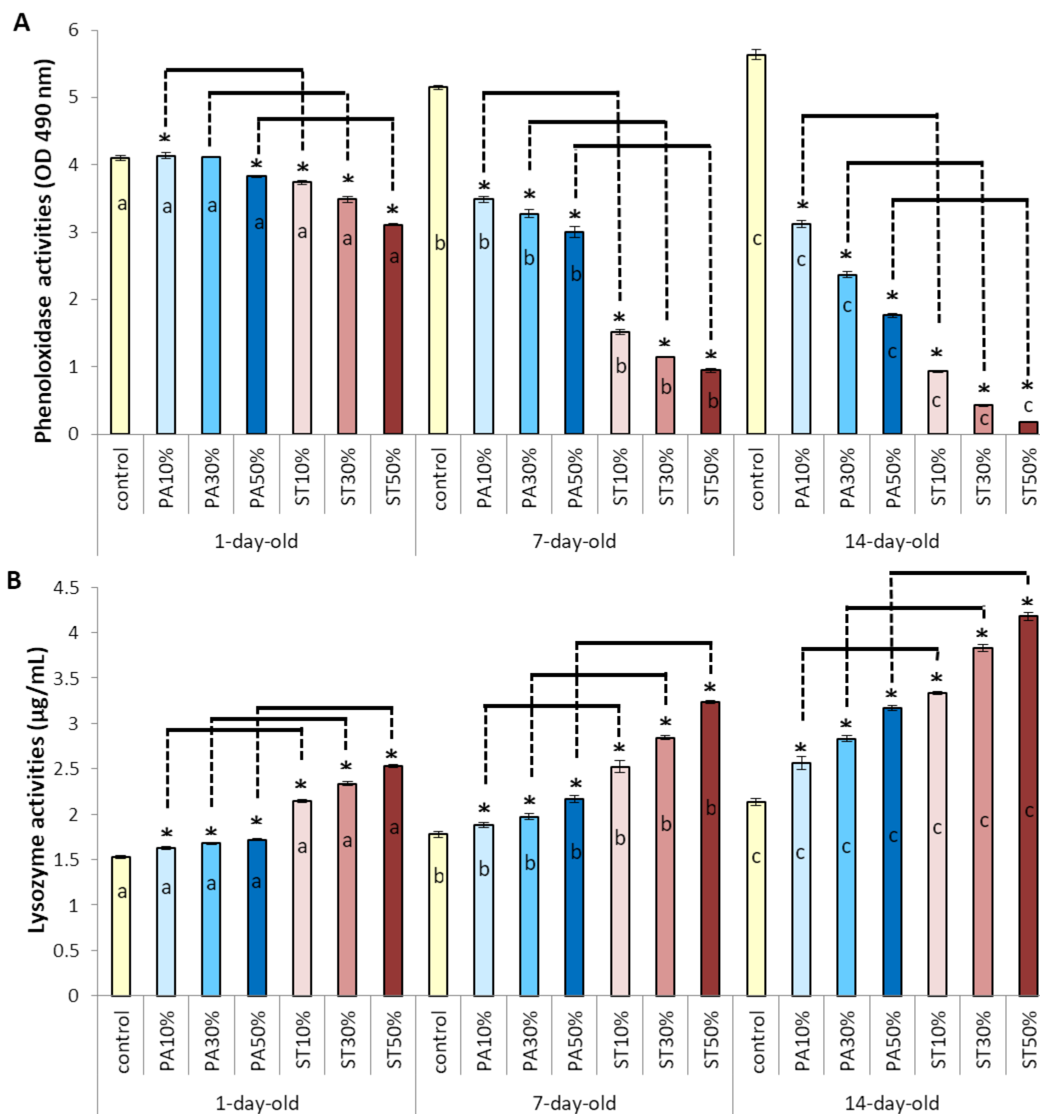

Fig. 1. Effect of adulteration of wax foundation with paraffin and stearin on phenoloxidase (A) and lysozyme (B) activities in the haemolymph of *A. mellifera* worker bees

PA10% – paraffin 10%, n = 30; PA30% – paraffin 30%, n = 30; PA50% – paraffin 50%, n = 30; ST10% – stearin 10%, n = 30; ST30% – stearin 30%, n = 30; ST50% – stearin 50%, n = 30; * – statistically significant difference within an age cohort between the control group and a group with a certain percentage of adulteration with paraffin or stearin (P-value ≤ 0.01); horizontal lines – statistically significant difference within an age cohort between an adulteration level of wax with paraffin and the same adulteration level of wax with stearin (P-value ≤ 0.01); a, b, c – statistically significant differences between age cohorts for the same adulteration level of wax and the same adulterant (P-value ≤ 0.01); bars and whiskers – standard deviation

Discussion

Phenoloxidase is a humoral protein with antimicrobial and antioxidant properties. Acting as a catalyst in the melanisation process (9, 23), it plays a key role in the immune responses of the honey bee. In the homeostatic state, PO exists as the zymogen prophenoloxidase. Activation of proPO occurs upon contact with a pathogen through a cascade of pattern recognition proteins, serine proteases and serine protease inhibitors (15). Our results show that adulteration of wax with stearin decreased PO activities in 1-, 7- and 14-day-old bees. It is worth noting here that this effect was delayed in time, because contact with a harmful substance in the preimaginal period resulted in a change in enzyme activities in adult bees. Most probably, the mechanism of stearin toxicity occurs in two steps. The first consists in the reaction of stearic acid with chitin, leading to chemical modifications of its functional groups (esterification of 2-hydroxyl groups and deacetylation of the N-acetyl amine group) (7). The stability of the insect cuticle is disturbed by the loosening of the structure, and consequently the damaged cuticle does not protect against water loss, mechanical damage or the penetration of pathogens into the host system. The second step is the permeation of stearic acid into the workers' haemolymph and subsequent inactivation of proPO by interaction of the acid with cells present in the haemolymph either directly by acting on this protein or by interaction with lysozyme. An increase in lysozyme activity may also indicate a decrease in the bees' immunity. In addition to being one of the antimicrobial peptides, lysozyme also acts as a proPO suppressor, which may explain the negative correlation between the increase in activity of this protein and the activity of PO.

The effect of PA seems to be less toxic compared to that of ST, but also statistically significant relative to the control group for PO activities. Our results indicate that development of preimaginal stages of worker bees in contact with this substance leads to a blockage of PO system activity. As shown by Ibrahim *et al.* (10, 11), paraffin can be a nutrient for bacteria. Presumably, even if these bacteria are not directly pathogenic to bees, the metabolites they secrete may act as PO inhibitors. This is supported by the decline in PO activity progressing with worker age. Decreased PO activity disturbs the defensive and regenerative abilities of the insect, and the increase in lysozyme activities observed by us in this case is a second line of humoral defence.

Most of the publications describing wax adulteration show changes in the composition of the components and/or the properties of such wax (17, 26, 33) and/or the brood development of honey bees surrounded by it (3, 5). This is the first publication regarding the impact of adulterated wax on the physiology of bees. We are the first to fill this knowledge gap and show how great the effects are on the functioning of the bee organism of the addition of stearin and, to a lesser extent, paraffin to natural wax. Our research indicates that it is highly probable that the more and more common adulteration of wax with paraffin and stearin, leading to dysregulation of phenoloxidase and lysozyme activities and most likely to immunodeficiency in worker bees, is a hitherto undiagnosed cause of the extensive losses of colonies that started in the late 20th century. Our suggestion is apparently consistent with the results of research by Chęć *et al.* (5), who showed that the addition of 10%, 30% or 50% stearin to wax foundations causes a decrease in brood survival by 67%, 87% or 92%, respectively, compared to broods developing in pure wax. This also explains why we could only include a maximum of 300 bees in one group. The remaining ones, as in the experiment of Chęć *et al.* (5), died at preimaginal stages of development. Therefore, it can be assumed, based on the results of these authors and our research, that the effects of using adulterated wax in a bee colony are complex – ranging from changes in the physicochemical properties of the foundations to changes in the amount of brood emergence and in the activities of enzymes that are key to the bees' immunity. Therefore, it is important to continue research on the effects of wax adulteration on the physiology of worker bees.

Conclusion

Adulteration of wax foundation with even a small amount of stearin or paraffin has negative effects on the functioning of the bee.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was funded by the University of Life Science in Lublin; grant no. LKE/S/64/2022/BIO and LKE/S/64/2023/BIO.

Animal Rights Statement: None required.

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